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Immunization of Mice with Pneumolysin Toxoid Confers a Significant Degree of Protection against At Least Nine Serotypes of *Streptococcus pneumoniae*

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Pneumolysin is the thiol-activated cytolysin produced by *Streptococcus pneumoniae*. Mice were immunized with a genetically engineered toxoid version of pneumolysin, which was derived from a serotype 2 pneumococcus. The toxoid carried the mutation Trp-433→Phe. Alum was used as the adjuvant. Immunized mice had significantly increased levels of anti-pneumolysin antibodies, principally immunoglobulin G1. Mice were challenged intraperitoneally or intranasally with 12 strains covering capsular serotypes 1 to 6, 7F, 8, and 18C. Following challenge, the survival rate and/or the time of death of nonsurvivors (survival time) was significantly greater than that of sham-immunized mice for all nine serotypes. However, differences in the degree of protection were noted between different strains. The route of challenge also appeared to influence the degree of protection. Nevertheless, the significant, albeit in some cases partial, protection provided against all nine pneumococcal serotypes supports the conclusion that pneumolysin toxoids warrant consideration for inclusion in a human vaccine.

Streptococcus pneumoniae is an important pathogen, being a principal cause of bacterial pneumonia, meningitis, and bacteremia. Morbidity and mortality rates due to these invasive diseases are high. The elderly and young are particularly at risk (2); an estimated five million children under 5 years of age die from pneumonia in developing countries each year (11). Although antimicrobial drugs have reduced mortality rates, therapy may be ineffective in high-risk groups unless given early in the course of infection (2). Furthermore, the number of drug-resistant strains of *S. pneumoniae* isolated appears to be increasing (24).

Existing polyvalent antipneumococcal vaccines are formulated from 23 capsular polysaccharides selected from the 84 serologically distinct types currently recognized. Although reported to be effective in some populations (22), these vaccines have two shortcomings. Firstly, the protection provided is serotype specific. This means that the combination of polysaccharides effective for one population may not be as effective for another in which the serotype prevalence is different. The serotypes covered in the vaccines account for 85 to 90% of serious pneumococcal infections in Europe and North America (12, 17, 21), but in several Asian countries the 23 vaccine serotypes account for as little as 63% of infections (12). In these countries the efficacy of the current vaccines may be substantially compromized.

A second shortcoming of the present vaccines is that the immunological properties of polysaccharides are such that they are poor immunogens, especially for infants, the elderly, and others most at risk (6, 18). This problem is being addressed by conjugation of the least-immunogenic polysaccharide types to protein carriers (1). However, the number of serotypes covered by polyvalent conjugate vaccines is likely to be much lower than 23, and so problems due to geographical variations in serotype prevalence will continue.

One strategy to overcome the problems associated with polysaccharide-based vaccines is the inclusion of pneumococcal proteins in a vaccine, either as protein carriers conjugated to polysaccharides and/or as protective immunogens in their own right (18). Pneumolysin, a pneumococcal thiol-activated toxin, is an attractive candidate protein antigen for inclusion in such a new vaccine. Virtually all clinical isolates of *S. pneumoniae* are known to produce pneumolysin (9). Pneumolysin is a protective immunogen; mice immunized with native pneumolysin are protected against subsequent challenge with virulent type 2 pneumococci (19). Although, native pneumolysin is unsuitable for inclusion in a human vaccine because of its toxic nature, pneumolysin derivatives of reduced toxicity developed by site-directed mutagenesis could be used (4). Mutated pneumolysin toxoids have been shown to be effective in protecting mice from challenge with virulent type 2 pneumococci (20). However, the ability of pneumolysin to protect against pneumococci independently of the capsular serotype has not been reported. Because of the conservation in the amino acid sequence (15), it has been suggested that inclusion of pneumolysin toxoids may overcome the serotype specificity of polysaccharide vaccines. To explore this possibility further, independent studies were set up in two centers, Adelaide, Australia, and Leicester, United Kingdom. We investigated the ability of immunization with pneumolysin toxoid to protect mice challenged, intraperitoneally or intranasally, with 12 *S. pneumoniae* strains covering nine different serotypes.

Bacterial strains. In Adelaide, all of the strains of *S. pneumoniae* used were clinical isolates from the Women's and Children's Hospital, Adelaide, South Australia, Australia, except for the type 3 strain GB05, which was a clinical isolate from The Royal Infirmary, Leicester, United Kingdom. In

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Leicester, *S. pneumoniae* serotypes 1, 3, and 18C were obtained from the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. The type 2 strain, NCTC 7466, was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom, and the type 7F strain was the clinical isolate also used in the Adelaide study. Pneumococci were serotyped by the Quellung reaction with antisera from Statens Serum-institut, Copenhagen, Denmark. Pneumococci were routinely cultured on blood agar plates (blood agar base containing 5% [vol/vol] horse blood) or in brain heart infusion broth (BHI) containing 20% (vol/vol) newborn calf serum (BHI-NCS).

Preparation of pneumolysin toxoid. The pneumolysin toxoid used in this study (PdB) has a reduced hemolytic activity of 0.05% that of the wild-type toxin, because of a Trp-433→Phe substitution, obtained by oligonucleotide-directed mutagenesis of the pneumolysin gene cloned from the type 2 strain NCTC 7466 (4). Toxoid was purified from *Escherichia coli* JM109[pJCP202] either by ion-exchange and gel filtration (20) in Adelaide or by hydrophobic interaction chromatography (16) in Leicester. Toxoid purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by staining with Coomassie brilliant blue R250, which indicated the presence of a single 52-kDa band accounting for at least 95% of the protein.

Immunization of mice. In the Adelaide study, male and female outbred, Quakenbush strain (Q/S) mice, 6 to 8 weeks old, were injected subcutaneously with 20 μg of PdB in 20% alum (Imject Alum; Pierce, Rockford, Ill.) in phosphate-buffered saline (PBS). Mice in the control group received 0.1 ml of alum in PBS. At 14-day intervals, the mice were given two additional injections. Blood samples were collected 7 days after the last injection. Sera were tested for the presence of antibodies to pneumolysin by double gel immunodiffusion and by enzyme-linked immunosorbent assay (ELISA) (8).

In Leicester, female MF1 outbred mice, ca. 30 to 35 g in weight, were obtained from Harlan Olac Ltd. (Shaw's Farm, Bicester, Oxfordshire, United Kingdom). Mice in the Freund's adjuvant-immunized groups were injected intraperitoneally with 20 μg of purified PdB in 0.1 ml of PBS–50% (vol/vol) glycerol emulsified with 0.1 ml of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Mo.). At 10-day intervals, the mice were given two additional injections of 20 μg of purified PdB in PBS–50% (vol/vol) glycerol emulsified with Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, Mo.). Mice in the Freund's control groups received the same course of injections of PBS-glycerol-adjuvant, but without PdB. Mice in the alum-immunized groups were injected intraperitoneally with 20 μg of purified PdB in 200 μl of alum adjuvant in a suspension of 8 mg of aluminum phosphate per ml in saline (National Institute of Public Health and Environmental Protection). Mice in the alum-immunized control groups were injected with 200 μl of alum adjuvant alone. At 10-day intervals, the mice were given two additional injections. Blood samples were taken before each injection and before challenge. Sera from mice were tested for the presence of antibodies to pneumolysin by ELISA (8). In the Leicester study, the immunoglobulin isotypes were identified by ELISA using a clonotyping system kit (Southern Biotechnology Associates, Birmingham, Ala.).

Preparation of challenge dose. In the Adelaide study, bacteria from fresh overnight cultures on blood agar were grown at 37°C to mid-log phase in meat extract broth containing 10% (vol/vol) horse serum (serum broth). Pneumococci were diluted as appropriate in fresh serum broth immediately before challenge, and viable counts were determined. In Leicester, BHIs (10 ml) were inoculated with four to five colonies taken from a fresh culture plate of mouse-passaged *S. pneumoniae* and incubated overnight at 37°C. Bacteria were harvested by centrifugation, resuspended in 1 ml of BHI-NCS, and then diluted with fresh BHI-NCS to give an A_{600} of 0.7. The culture was incubated at 37°C for 4 to 5 h. Viable colony counts were determined in triplicate on blood agar plates, and cultures were diluted as appropriate in fresh BHI-NCS. These samples were stored at −70°C. Pneumococci could be stored for at least 3 months at −70°C with no significant loss of viability. When required, the suspension was thawed slowly at room temperature, and bacteria were harvested by centrifugation before resuspension in sterile PBS.

Challenge of mice. In the Adelaide study, two weeks after the final injection, mice were challenged intraperitoneally with a 0.1-ml dose of pneumococci calculated to represent approximately 20 times the 50% lethal dose for each strain. The time of death of each mouse over the subsequent 14-day period was recorded. The experiment was ended after 14 days, and mice alive at this time were recorded as survivors.

In the Leicester study, mice were challenged intranasally and a different end point was used. One month after the final injection, mice were lightly anesthetized by intraperitoneal injection of 80 μl of Hypnorm (0.315 mg of fentanyl citrate per ml–10 mg of fluanisone per ml; Janssen Pharmaceuticals) diluted to a final concentration of 1 mg/ml. Twenty minutes after administration of the anaesthetic, mice were challenged with 50 μl of PBS containing the desired number of CFU of *S. pneumoniae*, administered into the nostrils. Challenged mice were kept warm until they had recovered consciousness (2 to 3 h). The mice were monitored for visible clinical symptoms for 14 days, at which point the experiment was ended. Mice that were alive at this point were considered to have survived the pneumococcal challenge. Mice that became moribund during the 14-day period were judged to have reached the end point of the assay. The time that the animal became moribund was recorded, and the animal was killed humanely. Data were analyzed by a one-tailed Mann-Whitney U test for median survival times and by a one-tailed χ^2 test for survival rates. Antibody data were analyzed by the Student t test.

Determination of hemolytic activities of challenge strains. To determine the total amount of pneumolysin produced by the various challenge organisms (as judged by hemolytic activity), each strain was cultured to late logarithmic phase (A_{600} of 1.0) in Todd-Hewitt broth with 5% (wt/vol) yeast extract and lysed by sonication (in Leicester) or with 0.2% (wt/vol) sodium deoxycholate (in Adelaide). The hemolytic activity of pneumolysin in each lysate was then determined as previously described (19), using human erythrocytes in Adelaide and sheep erythrocytes in Leicester.

The results from both studies (Tables 1 and 2) indicated some diversity in the levels of pneumolysin produced. In the strains used in Adelaide (Table 1), results ranged from 12 hemolytic units (HU) per ml of culture for the type 8 strain to 693 HU/ml for the type 5 strain. There was no relationship between the amount of active pneumolysin produced by these strains and virulence. However, interestingly, the type 5 strain, which produced the most pneumolysin activity, was less virulent than the type 8 strain, which produced the lowest amount of active toxin.

Among the strains used in Leicester (Table 2), the range of total activities was narrower than that for the Adelaide strains, varying from 140 to 640 HU/ml. As described above, no relationship was seen between the total hemolytic activity and virulence (Table 2). However, strain 18C, which produced the

TABLE 1. Protection against intraperitoneal challenge with *S. pneumoniae* strains of different serotypes elicited by immunization of Q/S mice with PdB

Type	HU[a]	Dose[b]	Median survival time (days)		P[c]	% Survival		P[d]
			Control	Immunized mice		Control	Immunized mice	
1	621	1×10^7	5.1	>14	<0.001	13	60	<0.005
3	335	1×10^5	3.0	3.8	<0.025	13	0	NS
4	585	4×10^3	1.8	>14	<0.003	7	73	<0.0005
5	693	2×10^8	5.5	9.3	<0.005	15	36	NS
6	424	1×10^7	3.9	>14	<0.001	7	67	<0.005
7F	221	2×10^7	1.8	7.9	<0.05	20	33	NS
8	12	4×10^3	1.2	1.5	<0.003	0	7	NS
18C	330	1×10^5	1.8	2.3	<0.03	0	14	NS

[a] Pneumolysin production expressed as hemolytic units per milliliter culture at A_{600} of 1.0.
[b] Challenge dose (CFU).
[c] Significance of difference, Mann-Whitney U test.
[d] Significance of difference, χ^2 test; NS, no significant difference.

lowest hemolytic activity, was found to require the highest dose of bacteria (10^7 CFU) to establish infection in mice.

Comparison of Freund's and alum adjuvants. Immunization with native pneumolysin, recombinant wild-type pneumolysin, or pneumolysoid PdB has previously been reported to protect against intranasal challenge with *S. pneumoniae* type 2 strain NCTC 7466 (19, 20). In these studies, the protein was administered in Freund's adjuvant (19, 20). Immunization and challenge experiments were performed to determine whether an equivalent protection was induced when pneumolysoid was administered in alum, an adjuvant approved for use in humans. Groups of 20 MF1 mice immunized with PdB in Freund's adjuvant or alum and control mice (sham immunized with the respective adjuvant) were challenged intranasally with a dose of 10^6 CFU of NCTC 7466. The survival time of each mouse was recorded. The median survival time was the same with both adjuvants: 3 days for controls and >14 days for the immunized groups ($P < 0.01$ for both groups). The survival rates were similar: 15% for controls with both adjuvants and 80 and 90% for the immunized groups with Freund's and alum adjuvants, respectively ($P < 0.01$ for all groups). Statistical analysis indicated that the protection afforded against strain NCTC 7466 by immunization with pneumolysoid PdB in alum was as great as that when PdB was administered in Freund's adjuvant.

Analysis of serum response to immunization with pneumolysoid. Serum samples were collected from mice before each immunization and challenge and analyzed by ELISA. All immunized mice had anti-pneumolysin antibody titers greater than those of control mice (Fig. 1). No significant difference in the mean anti-pneumolysin antibody response was found when PdB was given to MF1 mice with Freund's or alum adjuvant (Fig. 1). In Q/S mice, anti-pneumolysin titers in sera from animals immunized with PdB in alum were 250 times higher than those in sera from nonimmunized mice. Such levels of anti-pneumolysin antibodies were directly comparable to those from sera obtained in previous studies following immunization with PdB in Freund's adjuvant (20).

On completion of the course of immunization of MF1 mice, the serum samples taken from PdB-alum-immunized mice were analyzed by ELISA to identify the anti-pneumolysin immunoglobulin isotypes present. The results confirmed that the anti-pneumolysin antibody levels had been significantly increased ($P < 0.05$) in immunized mice from a mean of 0.07 mg/ml to a mean of 4.90 mg/ml (Fig. 2). The isotype analysis of the anti-pneumolysin antibodies present after immunization (Fig. 2), indicated that they were mainly from immunoglobulin classes immunoglobulin G1 (IgG1), IgG2a, and IgG2b at concentrations of 2.94, 1.14, and 0.05 mg/ml, respectively. Anti-pneumolysin antibodies of immunoglobulin classes IgA, IgM, and IgG3 were less than 0.1 mg/ml.

Intraperitoneal challenge of mice with *S. pneumoniae* strains. Groups of 15 immunized and nonimmunized Q/S mice were challenged intraperitoneally with the dose of each strain of pneumococcus shown in Table 1, and the survival times of the mice were recorded. The median survival time and overall survival rate for each group are also shown in Table 1. Statistical analysis indicated that the survival times of the mice

TABLE 2. Protection against intranasal challenge with *S. pneumoniae* strains of different serotypes elicited by immunization of MF1 mice with PdB

Type (strain)	HU[a]	Dose[b]	Median survival time (days)[c]		P[d]	% Survival		P[e]
			Control	Immunized mice		Control	Immunized mice	
1	160	4×10^6	3.0	3.9	<0.05	10	40	<0.01
2	640	1×10^6	3.2	>14	<0.01	6	72	<0.01
3	320	1×10^6	2.8	4.0	<0.01	10	33	<0.01
3 (GB05)	380	1×10^6	1.8	2.1	NS	0	0	NS
7F	140	2×10^6	2.9	>14	<0.01	0	70	<0.01
18C	260	1×10^7	3.2	>14	<0.01	5	85	<0.01

[a] Pneumolysin production expressed as hemolytic units per milliliter culture at A_{600} of 1.0.
[b] Challenge dose (CFU).
[c] Time at which mice became moribund, rather than death, was used as the end point in these experiments.
[d] Significance of difference, Mann-Whitney U test; NS, no significant difference.
[e] Significance of difference, χ^2 test.

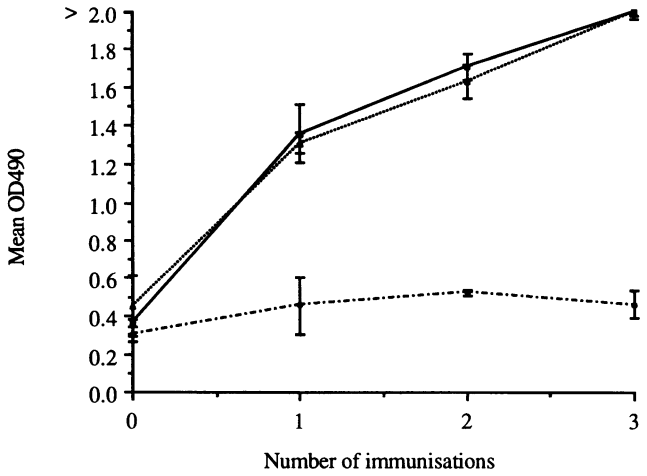

FIG. 1. Levels of total anti-pneumolysin immunoglobulin in serum of MF1 mice after vaccination with 20 μg of pneumolysoid PdB in Freund's adjuvant (.....), alum adjuvant (___), or PBS control (— · — · —), measured by ELISA. Each point represents the arithmetic mean for 10 mice; bars represent the standard errors of the means. OD490, optical density at 490 nm.

immunized with PdB were significantly increased with respect to those of the control mice with each of the eight challenge strains of pneumococcus. The percent survival rates, based on the number of mice alive 14 days after challenge, were also significantly increased for immunized groups challenged with strains belonging to serotypes 1, 4, and 6.

Intranasal challenge of mice with *S. pneumoniae* strains. Groups of 20 PdB-alum-immunized and control mice were inoculated intranasally with a dose of *S. pneumoniae* serotype 1, 2, 3, 7F, or 18C, which caused the control mice to become moribund in approximately 3 days (Table 2). The survival time of each mouse was recorded, and the median survival time and the overall survival rate are shown in Table 2. A significant increase in both the median survival times and survival rates of immunized mice was seen in each case except for type 3 strain GB05.

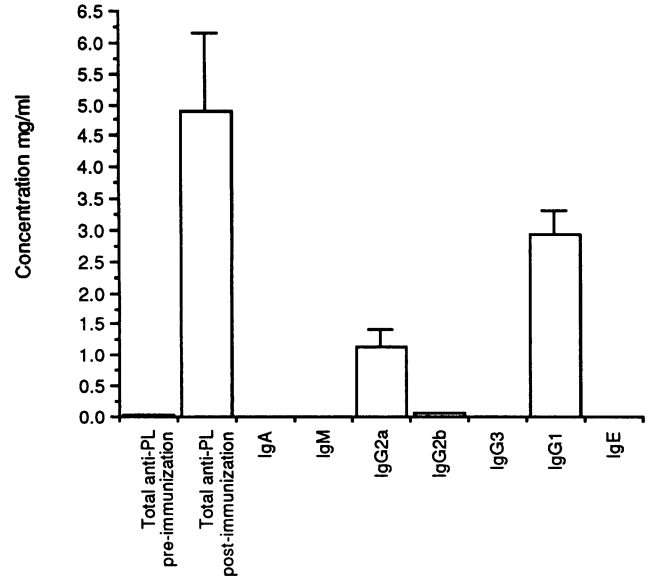

FIG. 2. Levels of anti-pneumolysin (PL) immunoglobulins of different isotypes in serum of MF1 mice after vaccination with 20 μg of pneumolysoid PdB in alum adjuvant, measured by ELISA. Each column represents the arithmetic mean for 10 mice; bars represent the standard errors of the means.

Previous studies have shown that immunization with various pneumococcal proteins provides a degree of protection against subsequent challenge with virulent pneumococci (13, 14, 19, 20, 25). Of these, pneumolysin and pneumococcal surface protein A (PspA) appear to provide superior protection (18). However, although immunization of mice with a truncated version of PspA provided a high degree of protection from challenge with virulent pneumococci producing a similar PspA antigenic type (25), studies with monoclonal antibodies have shown that significant antigenic variability exists (5) and may complicate the use of PspA in human vaccines.

The absence of significant amino acid sequence variation between pneumolysins from different serotypes (15) suggests that the problem with PspA does not apply to this immunogen. However, the capacity of pneumolysin toxoids to protect against a range of pneumococcal serotypes has not been demonstrated before. In the two studies described here, we have now demonstrated that immunization with PdB confers significant, although not necessarily complete, protection against strains covering several serotypes of *S. pneumoniae* in a model of either respiratory infection (intranasal challenge) or systemic infection (intraperitoneal challenge). The previously reported ability of the pneumolysin derivative PdB to protect immunized mice against type 2 *S. pneumoniae* (20) has also been confirmed.

However, although a statistically significant degree of protection was seen with each strain, the degree of protection given to mice sometimes was lower with particular strains and also varied with the route of infection. After intranasal challenge the degree of protection against the type 1 and type 3 strains was less than that against the other strains. When challenged intraperitoneally, there was less protection against the type 8 strain. A possible explanation for these observations is that the contribution that pneumolysin plays in the overall virulence of the pneumococcus in the type 1, 3, and 8 strains tested here may be somewhat less than that in the other strains tested. Other proteins may have a more prominent role in the virulence of these strains. Nevertheless, the statistically significant increase in survival time of mice challenged with types 1, 3, and 8 after immunization with pneumolysoid shows that pneumolysin does play a part in the pathogenesis of disease with these strains. It may be that the degree of protection provided against types 1, 3, and 8 and indeed other serotypes could be improved by combining PdB with additional pneumococcal proteins, and studies investigating this possibility are underway.

Comparison of the data from the two studies suggests that anti-pneumolysin prophylaxis is most effective when the pneumococci are in the lungs. The number of mice surviving to the end of the experiment was significantly higher for immunized mice given each of the serotypes intranasally, except for the GB05 strain. This was not always so when the strains were given intraperitoneally. Given that natural pneumococcal infection occurs via the respiratory route, it is fortunate that pneumolysoid acts best as a protective immunogen against pneumococci delivered by that route. We have shown in previous experiments that pneumolysin can directly contribute to disease in the lungs; the purified toxin will, by itself, produce the pathological features of pneumococcal pneumonia when instilled into rat lungs (7). We do not know how pneumolysin

contributes to the pathology during systemic infection, but the observations here suggest that it may be relatively less important than when pneumococci are in the lungs.

The lack of protection afforded against the type 3 strain GB05 when given intranasally shows that pneumolysin is not a panacea as a vaccine and reinforces the suggestion that additional pneumococcal immunogens should be explored for use in conjunction with the pneumolysin toxoid. The absence of protection is not explained solely by the presence of the type 3 polysaccharide since significant protection was seen against the other type 3 strain. The presence of the same capsular polysaccharide in two isolates of pneumococcus does not mean that they are the same clone. Recent data (23) have shown that the same capsular serotype can be distributed between completely different pneumococcal clones.

The lack of protection against intranasal challenge does not indicate that pneumolysin has no part to play in the virulence of the GB05 strain, because immunization did confer significant protection against intraperitoneal challenge. Moreover, it has been shown previously that inactivation of the pneumolysin gene in this strain by insertional mutagenesis results in a significant decrease in virulence, as judged by the intraperitoneal 50% lethal dose (3). Nevertheless, we conclude that other, unidentified factors do play an important role in the virulence of the GB05 strain. The influence of this factor(s) appears to be more pronounced when the bacteria are in the lungs. Thus, there appear to be several virulence factors in the pneumococcus, each of which has a different degree of influence on the overall virulence in different strains and whose influence on virulence varies with the environment of the pneumococcus. Pneumolysin is one of these virulence factors and as such is a legitimate target for immunoprophylaxis. However, other, as yet unidentified, factors also appear to exist and could also be vaccine targets.

No direct relationship between pneumolysin hemolytic activities from the strains used and their virulence was seen. This does not mean that pneumolysin is not a virulence factor for *S. pneumoniae*; rather it suggests that production of a minimum threshold level of pneumolysin is sufficient for virulence. Nevertheless, any variation in virulence that may exist between the strains studied here may be a consequence of other, undefined factors. Furthermore, little is known about the regulation of pneumococcal virulence genes, and it is possible that the level of expression of the pneumolysin gene in vitro differs from that in vivo. An analogous situation has been reported for strains of *Listeria monocytogenes*, which also produce a related thiol-activated hemolysin. In this species hemolysin production is essential for virulence, but the level of hemolysin production is not directly proportional to the severity of infections in mice (10).

Conclusions. The results of the two studies presented here add to the body of evidence that immunization with PdB confers non-serotype-specific protection against *S. pneumoniae*. We have shown that PdB elicits a degree of protection against at least 12 strains of *S. pneumoniae* representing nine serotypes. Pneumolysin toxoids therefore should be considered for inclusion in improved pneumococcal conjugate vaccines for use in humans.

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